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Increased expression of hepatocyte growth factor (HGF) and its receptor Met has been identified as a possible independent indicator of recurrence in breast cancer patients. Dr. Elliott's laboratory has previously shown increased expression of HGF and Met in regions of invasive human breast cancer. In addition, we have found that breast carcinoma cell lines frequently express HGF and Met, whereas most nonmalignant epithelial cell lines express Met but not HGF. Together these results suggest that establishment of an autocrine HGF loop in carcinoma cells, and the change in the regulation of HGF expression is an important indicator of breast cancer progression. I have shown that in one breast carcinoma cell line MCF10A1 T3B expressed additional forms of HGF. This may represent one mechanism by which breast cancer cells regulate the pericellular level of HGF. I have identified 2 Stat3 binding sites on the *HGF* promoter, which are required for responsiveness of *HGF* transcription to the level of c-Src kinase activity. Changes in c-Src kinase activity affect Stat3 activity through its tyrosine phosphorylation and DNA binding affinity. Ras activity can also regulate *HGF* transcription in breast carcinoma cells, and preliminary evidence suggested phosphatidylinositol 3-kinase is a downstream target of this pathway.

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Background:

HGF, Met, c-Src and Breast Cancer Progression

Hepatocyte growth factor (HGF) is a multifunctional cytokine. Through binding to its receptor (Met), HGF can induce cell growth (42), differentiation (31) and motility (15). It has been shown that both HGF and Met are essential to embryo development. Mice lacking HGF expression result in impaired placenta, and lethality before birth (44) while homozygous deletion of the *met* gene causes under-development of the limb buds and intra-uterine death (2). Increased expression of HGF and Met has also been identified as a possible independent indicator of recurrence in breast cancer patients (46). Our laboratory has previously shown increased expression of HGF and Met in regions of invasive human breast cancer (47). In addition, we have found that breast carcinoma cell lines frequently express HGF and Met, whereas most nonmalignant epithelial cell lines express Met but not HGF. HGF also stimulates anchorage-independent survival of carcinoma cells (40). Together, these results suggest that establishment of an autocrine HGF loop in carcinoma cells may promote mammary tumor progression.

A number of signalling molecules, such as c-Src (40), Ras (19), Grb2 (13) and phosphatidylinositol (PI) 3-kinase (6;38), have been shown to be part of the HGF/Met signalling pathway. Activation of Met through binding of HGF, causes auto-phosphorylation of tyrosine residues in the cytoplasmic tail of the receptor tyrosine kinase (36;37). These tyrosine phosphorylations recruit cytoplasmic signalling molecules and cause their activation. The c-Src non-receptor tyrosine kinase is expressed in many cell types, and its activity is activated in response to HGF and binding to Met (40). Activation of c-Src kinase can lead to increased expression of many genes, including growth factors such as vascular endothelial growth factor (VEGF) (32;33) and parathyroid hormone-related peptide (23). Increased activation of the tyrosine kinase c-Src occurs in many cancer cells, and c-Src plays a critical role in breast cancer. In a transgenic mouse model, over-expression of an activated form of c-Src induces mammary hyperplasia (50). Furthermore, c-Src kinase is activated during polyoma middle T- induced mammary tumorigenesis in transgenic mice. However, expression of polyoma middle T in *c-src*$^{-/-}$ background mice fails to cause tumor formation (17). Together, these results show that c-Src is necessary, but not sufficient, for mammary tumorigenesis.

Hypothesis and Objectives

The overall hypothesis of this laboratory is that HGF is a critical survival factor in breast carcinoma cells during metastasis. Specifically, establishment of an HGF autocrine loop in breast carcinoma cells is pivotal in the transition from non-malignant to malignant epithelial growth. The change in the regulation of HGF expression would therefore be an important indicator of breast cancer progression. Moreover, by understanding the signalling pathways that regulate HGF expression, therapeutic treatment based on these approaches may be developed towards breast cancer progression and metastasis. The main objectives of my projects are:

1) To examine the transcriptional and post-transcriptional regulation of HGF expression in breast carcinoma cells; and
2) To determine the role of signalling molecules such as c-Src and PI-3 kinase in HGF expression in breast carcinoma cells.

Progress and Results

Objective 1

A) Transcriptional Control

To determine the expression of HGF under the effect of different cytokines, I used the mouse mammary carcinoma cell line SP1, which co-expresses HGF and tyrosine-phosphorylated Met (12;41). I extracted total RNA from SP1 cells exposed to various cytokines and performed reverse transcription coupled polymerase chain reaction (RT-PCR) to quantitate the amount of *HGF* mRNA in the samples. Primers were designed in Dr. Elliott's laboratory to amplify specifically mouse and human HGF but not its homologue MSP (Figure 1). Primers specific for a constitutively expressed gene -glucuronidase (GUS B) were used as an internal control. In the previous report I have demonstrated the use of RT-PCR as a reliable method of determining *HGF* mRNA level in cells. Preliminary experiments suggested that growth factors such as EGF and TNFα have positive effect on HGF mRNA level while TGF-β1 reduced the amount of HGF mRNA in SP1 cells (data not shown). Experiments are in progress to determine some of the factors (such as other cytokines) that determine *HGF* mRNA level in breast carcinoma cells using semi-quantitative RT-PCR and other methods.

B)Post-Transcriptional Control

To study HGF expression at the protein level, I have shown in the previous report the characterization of two antibodies specific for the N-terminal portion of HGF that were developed in Dr. Elliott's laboratory as well as an antibody against full length HGF (provided by Genentech). These antibodies were used to identify HGF protein expressed by various breast carcinoma cell lines. I have identified several lower molecular weight bands immunoreactive with anti-HGF antibody in at least one of the breast carcinoma cell lines examined (MCF10A1 T3B) (data not shown). These immunoreactive bands may be novel HGF isoforms or results of specific degradation of HGF. Experiments are in progress to determine some of the proteases involved in the post-transcriptional regulation of HGF expression in breast carcinoma cells.

Objective 2

A) c-Src Non-receptor Tyrosine Kinase

To study the regulation of HGF expression in breast carcinoma cells, I used the mouse mammary carcinoma cell line SP1. Semi-quantitative RT-PCR was performed to determine the levels of *HGF* mRNA in SP1 cells. We first examined the dose-dependent effect of an inhibitor of c-Src family kinases, PP2 (18). Total RNA was isolated from SP1 cells treated with different concentrations of PP2 and used for cDNA synthesis by reverse transcription. Relative *HGF* mRNA levels were determined by RT-PCR using HGF-specific primers, and each sample was normalized to the expression of a house keeping gene -glucorunidase (GUS B) (20;30). The results showed that the PP2 inhibitor reduced *HGF* mRNA level in a dose-dependent manner to a maximum of 40% of untreated cells (Fig. 1A). To assess the effect of c-Src kinase activity on *HGF* mRNA expression, RT-PCR analysis was carried out on RNA extracted from SP1 cells expressing the different c-Src mutants, or treated with the PP2 inhibitor. Expression of the dominant negative SRC-RF (K295K Y527F) mutant or treatment with PP2 reduced the *HGF* mRNA level in SP1 cells by approximately 60%. Conversely, expression of the constitutively active c-Src mutant (SRC-Y527F) increased *HGF* mRNA expression by about two-fold.

In a parallel approach, the level of secreted HGF protein was compared in conditioned media collected from the same cells and under the same conditions described in Figure 3A. Dr. Elliott's laboratory has previously shown that HGF is a Cu (II) binding protein, and can be purified from conditioned media using copper (II) affinity chromatography (39) and analyzed on a denaturing SDS-

PAGE gel (Fig. 3B). Using this method, I showed that expression of the dominant negative SRC-RF mutant or treatment with PP2 significantly decreased the amount of HGF protein secreted by SP1 cells. In contrast, expression of activated c-Src showed an increased amount of secreted HGF protein. Together these data suggests that HGF expression (both at the mRNA and protein levels) is regulated by c-Src kinase activity.

In addition, I examined the level of transcription of the *HGF* gene using a reporter plasmid. A plasmid containing a 2.7 kb fragment 5' of the *HGF* transcriptional start site ligated to the firefly luciferase gene was transiently transfected into SP1 cells. Bell *et al.* (1)has previously shown that this 2.7 kb fragment of the *HGF* promoter contains all the necessary sequence to direct HGF expression and mimics the expression pattern of the endogenous *HGF* gene in transgenic mice. Transient expression studies in SP1 cells further support the notion that c-Src kinase activity is required for *HGF* transcription. Expression of activated c-Src (SRC Y527F) induces *HGF* promoter activity while expression of the dominant negative c-Src (SRC RF) reduces *HGF* promoter activity (Figure 9). To further understand the mechanism of regulation of HGF expression by c-Src kinase, I constructed a series of deletion mutants of the *HGF* promoter fused to luciferase and transiently transfected these into SP1 cells and also co-expressing activated or dominant negative forms of c-Src (Figure 10). Using this approach, I have located one region responsive to increased c-Src kinase activity. Deletion of this region and another region between -274 and -70 eliminated all the response of *HGF* promoter to activated c-Src, suggesting that this region is important in c-Src mediated *HGF* transcription. Similarly in my previous report I have shown that in Cos-1 fibroblast cells this region (-254 to –70) is also important in c-Src responsiveness of the *HGF* promoter (data not shown). The region, which is required for c-Src responsiveness in breast carcinoma cells, contains putative binding sites for Stat3 protein (Figure 11). Stat3 has been shown to activate transcription in response to increased c-Src kinase activity (43;48). In breast development, Stat3 is activated by c-Src leading to tubule formation (3). Therefore, I investigated whether Stat3 is involved in the regulation of HGF expression in response to c-Src. A reporter plasmid containing the -2.7 kb full length *HGF* promoter was co-transfected with a constant amount of the SRC-Y527F, and varying amounts of Stat3, expression plasmids. Expression of activated c-Src (SRC-Y527F) alone increased *HGF* transcription by about 2-fold (Figure 5A). Likewise, expression of Stat3 alone increased *HGF* transcription by about 2-fold, and maintained a plateau value with even 0.05 g of plasmid DNA. However, in cells co-expression both the activated c-Src mutant and increasing amounts of Stat3, *HGF* transcription increased up to 5-fold. This result indicates that there is a co-operative effect between c-Src kinase activity and Stat3 protein in the regulation of *HGF* transcription.

The nonmalignant mammary epithelial cell line, HC11, shows at least a15-fold lower level of *HGF* transcription and no detectable HGF protein compared to SP1 carcinoma cells (data not shown). We therefore determined whether co-expression of c-Src and Stat3 can activate *HGF* transcription in HC11 cells. Expression of activated c-Src induced expression by about 4 fold (Figure 5B). In contrast to SP1 cells, expression of Stat3 alone in HC11 cells did not significantly induce *HGF* transcription. However, when activated c-Src and Stat3 were co-expressed, *HGF* transcription was synergistically induced. These results suggest that increased c-Src kinase activity and Stat3 expression can over-ride the repression of HGF transcription in nonmalignant mammary epithelial cells.

To determine whether the c-Src responsive region of the *HGF* promoter is involved in the observed co-operative effect between c-Src and Stat3, the transcriptional activity of a mutant *HGF* reporter lacking the c-Src responsive region (HGF-luc) was compared to that of the full length (2.7 HGF-luc) *HGF* reporter. Each reporter construct was transfected into SP1 cells alone, or in combination with Stat3, and the activated c-Src (SRC-Y527F) mutant, expression plasmids. Expression of the activated c-Src mutant induced activation of the full length *HGF* promoter, but not of the deletion mutant (HGF-luc) (Figure 6). Similarly, Stat3 expression increased the activity of the full length *HGF* promoter, and only marginally affected that of the deletion mutant (HGF-luc); this result suggests that Stat3 activates the *HGF* promoter. The level of induction due to Stat3 expression is even higher than that due to activated c-

Src alone. This effect is probably due to a limiting amount of endogenous Stat3 in SP1 cells. When both Stat3 and activated c-Src were co-expressed, *HGF* promoter activity in the full length construct was strongly induced; this effect was not seen in the deletion mutant (HGF-luc). These results showed that Stat3 in cooperation with c-Src kinase can activate *HGF* promoter, and this activation is completely dependent on the presence of these Stat3 binding sites and implies a role of Stat3 as a downstream effector of c-Src kinase.

Stat3 has been shown to be regulated by both tyrosine and serine phosphorylations (27;45;51). Although there is no direct evidence that Stat3 is phosphorylated directly by c-Src, some reports suggest that c-Src and Stat3 interact physically (7). Therefore, it is possible that c-Src regulates Stat3 through tyrosine phosphorylation. Using antibody specific for the tyrosine phosphorylated form of Stat3 (Y705), I showed that expression of a dominant negative form of c-Src reduced the level of tyrosine phosphorylation of Stat3 in SP1 cells and the expression of constitutively active c-Src mutant had the opposite effect (Figure 9). In addition, we found that the formation of DNA-protein complex with the two Stat3 binding sites (-110 and –149) in the c-Src responsive elements was dependent on the level of c-Src kinase activity in the cells. Incubation of nuclear extracts from SP1 cells expressing activated c-Src mutant has more binding activity than that of untransfected SP1 cells. SP1 cells expressing the dominant negative c-Src mutant has the opposite effect (Figure 10). The binding was specific because addition of unlabelled oligonucleotides with unrelated sequence (NS) cannot eliminate the binding while unlabelled oligonucleotides corresponding to the *HGF* promoter Stat3 bindning sites (-110 or –149) can efficiently compete for binding (Figure 10). Supershift studies using antibodies against specific Stat proteins allowed us to identify Stat3, but not other Stat proteins, as a component of the DNA-protein complex (Figure 11). Addition of antibodies against Stat1 (data not shown), Stat5A or Stat5B cannot "supershift" the DNA binding complex. Only anti-Stat3 antibody can further retard the mobility of the DNA-protein complex on the gel, suggesting that Stat3 is part of the DNA-protein complex. Other Stat proteins were ruled because they were not expressed in mammary tissues. Together, these observations suggest that c-Src kinase may regulate Stat3-dependent transcriptional activity through direct or indirect tyrosine phosphorylation of Stat3 resulting in increased DNA-binding ability.

B) Ras and Phosphatidylinositol 3-kinase

Ras is an important signal transduction molecule that integrates signals from various stimuli and activates a variety of downstream signalling pathways (14;26). Ras can activate the MAPK pathway involving Raf and ERK (29), as well as the PI 3-kinase pathway (26), which is required for cell survival (38). I demonstrated that a farnesyltransferase inhibitor B956 (5;24), which can inhibit Ras family protein function, can affect the level of *HGF* mRNA in breast carcinoma cells (Figure 12). Using an activated Ras mutant (Ras V12), I showed that increased Ras activity can induce *HGF* transcription. To further dissect the pathway that is involved, I transfected various activated Ras mutants with the HGF-luciferase reporter into SP1 cells. Each of these activated Ras mutants has the activating V12 mutation as well as a second site mutation that selectively activates individual downstream pathways. Ras V12 E38 mutant selectively activates the Raf/ERK pathway (22) while the Ras V12 C40 activates only the PI 3-kinase pathway (25). Interestingly, expression of the Ras V12 E38 mutant has little effect on *HGF* transcription while Ras V12 C40 can efficiently up-regulate *HGF* transcription, suggesting that PI 3-kinase activity is important in regulating *HGF* transcription (Figure 13). This is further supported by the use of the PI 3-kinase inhibitor, LY294002 (26;49). Addition of the inhibitor reduces *HGF* transcription in a dose-dependent manner (Figure 14), reminiscent of the treatment with the Ras inhibitor B956. Further experiments are in progress to determine the region of *HGF* promoter that is responsive Ras/PI 3-kinase activity and the upstream pathway that is responsive to the activation of Ras and PI 3-kinase.

Conclusions

Studies of *HGF* transcription have revealed that HGF expression is under the regulation of c-Src tyrosine kinase. c-Src kinase has been shown to regulate the expression of other growth factors such as VEGF. In the case of VEGF, c-Src regulates expression through the transcription factor HIF-1 (21). I have identified a region on the *HGF* promoter that is responsive to increased c-Src kinase activity in both breast carcinoma cells and fibroblast cells. The pathway by which c-Src kinase regulates HGF expression involves the transcription factor, Stat3. Co-expression studies confirms that Stat3 can enhance the c-Src responsiveness of the *HGF* promoter and co-expression of activated c-Src and Stat3 can direct HGF transcription even in nonmalignant mammary epithelial cells, which do not express HGF. Electrophoretic mobility shift assays and super-shift assays revealed that Stat3 binds to and activates the activity of the *HGF* promoter and this is determined by the kinase of c-Src, which induces tyrosine phosphorylation of Stat3 as well as DNA binding activity of Stat3. These results suggest a similar regulation pattern between fibroblast cells that express HGF. It is possible that during epithelial-mesenchymal transition, epithelial cells acquire different genetic mutations leading to the activation of c-Src kinase. For example, increased expression and activity of the EGF-like receptor tyrosine kinase, HER2/Neu, in breast carcinoma cells has been shown to activate c-Src kinase (11;35). Activation of c-Src, in turn, might lead to a de-repression of HGF expression, giving these cells growth advantage compared to non-transformed epithelial cells. This step may be an important initial step in tumorigenesis. Experiments are underway to establish mammary epithelial cell line over expressing activated c-Src and Stat3. These cell lines will help to elucidate the hypothesis of whether acquisition of HGF expression in mammary epithelial cells (driven by increased activities of c-Src and Stat3) can "force" nonmalignant cells to become more cancer-like. Anchorage-independent growth, ascini formation in three-dimensional culture and other phenotypes will be examined.

Furthermore, PI 3-kinase and Ras are signal transduction molecules that regulate HGF expression in breast carcinoma cells. Since Ras mutations are frequently found in cancer cells (4;9;10;28) and PI 3-kinase activity is important in cell survival under anchorage independent conditions (38), these molecules may contribute greatly to breast cancer progression and one of the ways Ras and PI 3-kinase enhance breast cancer progression can be through the increased expression of HGF. Therefore, the role of these molecules in HGF expression are being further examined.

Finally to study the role of HGF expression in breast cancer progression *in vivo*, I am planning to construct a transgenic mouse strain carrying a reporter gene (-galactosidase) fused to the HGF promoter. This mouse strain will be crossed with another transgenic mouse strain that habours the MMTV LTR-driven expression of polyoma middle T protein (8). Expression of polyoma middle T protein under these conditions will be limited to breast epithelial cells and therefore, will induce breast tumors (16;34). Expression of HGF can be monitored by the expression of β-galactosidase as breast tumor progresses in the transgenic mice. Further backcrosses with other strains defective in signalling molecules (e.g. $src^{-/-}$) will be carried out to test the *in vivo* relevance of specific signalling pathways in HGF expression *in vivo*. This will reveal the stage of breast tumor progression at which HGF expression is up-regulated.

These studies will provide insight to the de-regulation of HGF expression in breast carcinoma cells. With the latest advances in specific tyrosine kinase inhibitors for cancer therapy, the studies of signalling molecules regulating HGF expression may provide novel targets for treatment.

References

1. Bell, A.W., J.G. Jiang, Q. Chen, Y. Liu, and R. Zarnegar. The upstream regulatory regions of the hepatocyte growth factor gene promoter are essential for its expression in transgenic mice. *Journal.of.Biological.Chemistry.1998.Mar.20.* 273:6900-6908.

2. Bladt, F., D. Riethmacher, S. Isenmann, A. Aguzzi, and C. Birchmeier. 1995. Essential role for the c-met receptor in the migration of myogenic precursor cells into the limb bud. *Nature* 376:768-771.

3. Boccaccio, C., M. Ando, L. Tamagnome, A. Bardelli, P. Michieli, C. Battistini, and P. Comoglio. 1998. Induction of epithelial tubules by hepatocyte growth factor depends on the STAT pathway. *Nature* 391:285-288.

4. Bos, J.L. 1900. The ras gene family and human carcinogenesis. *Mutation.Res. 1988.May.* 255-271.

5. Buss, J.E. and J.C.J. Marsters. Farnesyl transferase inhibitors: the successes and surprises of a new class of potential cancer chemotherapeutics. *Chemistry & Biology.1995.Dec.* 2:787-791.

6. Cantley, L.G. and L.C. Cantley. 1995. Signal transduction by the hepatocyte growth factor receptor, c-*met*: Activation of the phosphatidylinositol 3-kinase. *J.Am.Soc.Nephrol.* 5:1872-1881.

7. Cao, X., A. Tay, G.R. Guy, and Y.H. Tan. 1996. Activation and association of Stat3 with Src in v-Src-transformed cell lines. *Molecular.& Cellular.Biology.* 16:1595-1603.

8. Cardiff, R.D. and W.J. Muller. 1993. Transgenic mouse models of mammary tumorigenesis. *Cancer Surv.* 16:97-113.

9. Chien, C.H. and S.N. Chow. Point mutation of the ras oncogene in human ovarian cancer. *DNA & Cell Biology.1993.Sep.* 12:623-627.

10. Clark, G.J. and C.J. Der. Aberrant function of the Ras signal transduction pathway in human breast cancer. *Breast Cancer Research & Treatment.1995.Jul.* 35:133-144.

11. Degen, S.J., L.A. Stuart, S. Han, and C.S. Jamison. 1991. Characterization of the mouse cDNA and gene coding for a hepatocyte growth factor-like protein: expression during development. *Biochemistry* 30:9781-9791.

12. Elliott, B., A. Ostman, B. Westermark, and K. Rubin. 1992. Modulation of growth factor responsiveness of murine mammary carcinoma cells by cell matrix interactions: correlation of cell proliferation and spreading. *J.Cell.Physiol.* 152:292-301.

13. Fixman, E.D., M. Holgado-Madruga, L. Nguyen, D.M. Kamikura, T.M. Fournier, A.J. Wong, and M. Park. 1997. Efficient cellular transformation by the Met oncoprotein requires a functional Grb2 binding site and correlates with phosphorylation of the Grb2-associated proteins, Cbl and Gab1. *J.Biol.Chem.* 272:20167-20172.

14. Fuller, S.J., S.G. Finn, J. Downward, and P.H. Sugden. Stimulation of gene expression in

neonatal rat ventricular myocytes by Ras is mediated by Ral guanine nucleotide dissociation stimulator (Ral.GDS) and phosphatidylinositol 3-kinase in addition to Raf. *Biochemical Journal 1998.Oct.15.* 335:241-246.

15. Gherardi, E., J. Gray, M. Stoker, M. Perryman, and R. Furlong. 1989. Purification of scatter factor, a fibroblast-derived basic protein that modulates epithelial interactions and movement. *Proc.Natl.Acad.Sci.USA* 86:5844-5848.

16. Guy, C.T., R.D. Cardiff, and W.J. Muller. 1992. Induction of mammary tumors by expression of polyomavirus middle T oncogene: a transgenic mouse model for metastatic disease. *Mol.Cell.Biol.* 12:954-961.

17. Guy, C.T., S.K. Muthuswamy, R.D. Cardiff, P. Soriano, and W.J. Muller. 1994. Activation of the c-Src tyrosine kinase is required for the induction of mammary tumors in transgenic mice. *Genes Dev.* 8:23-32.

18. Hanke JH, Gardner JP, Dow RL, Changelian PS, Brissette WH, Weringer EJ, Pollok BA, and Connelly PA. 1996. Discovery of a novel, potent, and Src family-selective tyrosine kinase inhibitor. Study of Lck- and FynT-dependent T cell activation. *J.Biol.Chem.* 271:695-701.

19. Hartmann, G., K.M. Weidner, H. Schwarz, and W. Birchmeier. 1994. The motility signal of scatter factor/hepatocyte growth factor mediated through the receptor tyrosine kinase met requires intracellular action of Ras. *J.Biol.Chem.* 269:21936-21939.

20. Ivanchuk, S.M., S.M. Myers, and L.M. Mulligan. Expression of RET 3' splicing variants during human kidney development. *Oncogene 1998.Feb.26.* 16:991-996.

21. Jiang, B.H., F. Agani, A. Passaniti, and G.L. Semenza. 1997. V-SRC induces expression of hypoxia-inducible factor 1 (HIF-1) and transcription of genes encoding vascular endothelial growth factor and enolase 1: involvement of HIF-1 in tumor progression. *Cancer Research.* 57:5328-5335.

22. Joneson, T., M.A. White, M.H. Wigler, and D. Bar-Sagi. Stimulation of membrane ruffling and MAP kinase activation by distinct effectors of RAS. *Science 1996.Feb.9.* 271:810-812.

23. Karaplis, A.C., S.K. Lim, H. Baba, A. Arnold, and H.M. Kronenberg. 1995. Inefficient membrane targeting, translocation, and proteolytic processing by signal peptidase of a mutant preproparathyroid hormone protein. *J.Biol.Chem.* 270:1629-1635.

24. Kato, K., A.D. Cox, M.M. Hisaka, S.M. Graham, J.E. Buss, and C.J. Der. Isoprenoid addition to Ras protein is the critical modification for its membrane association and transforming activity. *Proc.Natl.Acad.Sci. U.S.A.1992.Jul.15.* 89:6403-6407.

25. Khosravi-Far, R., M.A. White, J.K. Westwick, P.A. Solski, M. Chrzanowska-Wodnicka, L. van Aelst, M.H. Wigler, and C.J. Der. Oncogenic Ras activation of Raf/mitogen-activated protein kinase-independent pathways is sufficient to cause tumorigenic transformation. *Molecular.& Cellular.Biology.1996.Jul.* 16:3923-3933.

26. Khwaja, A., P. Rodriguez-Viciana, S. Wennstrom, P.H. Warne, and J. Downward. Matrix adhesion and Ras transformation both activate a phosphoinositide 3-OH kinase and protein kinase

B/Akt cellular survival pathway. *EMBO Journal 1997.May.15.* 16:2783-2793.

27. Kuroki, M. and J.T. O'Flaherty. Extracellular signal-regulated protein kinase (ERK)-dependent and ERK-independent pathways target STAT3 on serine-727 in human neutrophils stimulated by chemotactic factors and cytokines. *Biochemical Journal 1999.Aug.1.* 341:691-696.

28. Lu, J., C. Jiang, S. Fontaine, and H.J. Thompson. ras may mediate mammary cancer promotion by high fat. *Nutrition.& Cancer 1995.* 23:283-290.

29. Marshall, M. 1995. Interactions between Ras and Raf: Key regulatory proteins in cellular transformation. *Mol.Reprod.Dev.* 42:493-499.

30. Miller, R.D., J.W. Hoffmann, P.P. Powell, J.W. Kyle, J.M. Shipley, D.R. Bachinsky, and W.S. Sly. Cloning and characterization of the human beta-glucuronidase gene. *Genomics 1990.Jun.* 7:280-283.

31. Montesano, R., K. Matsumoto, T. Nakamura, and L. Orci. 1991. Identification of a fibroblast-derived epithelial morphogen as hepatocyte growth factor. *Cell* 67:901-908.

32. Mukhopadhyay, D., L. Tsiokas, and V.P. Sukhatme. 1995. Wild-type p53 and v-Src exert opposing influences on human vascular endothelial growth factor gene expression. *Cancer Res.* 55:6161-6165.

33. Mukhopadhyay, D., L. Tsiokas, X.M. Zhou, D. Foster, J.S. Brugge, and V.P. Sukhatme. 1995. Hypoxic induction of human vascular endothelial growth factor expression through c-Src activation. *Nature* 375:577-581.

34. Muller, W.J. 1991. Expression of activated oncogenes in the murine mammary gland: transgenic models for human breast cancer. [Review]. *Cancer & Metastasis Reviews* 10:217-227.

35. Olayioye, M.A., I. Beuvink, K. Horsch, J.M. Daly, and N.E. Hynes. ErbB receptor-induced activation of stat transcription factors is mediated by Src tyrosine kinases. *Journal of Biological Chemistry 1999.Jun.11.* 274:17209-17218.

36. Park, M., M. Dean, C.S. Cooper, M. Schmidt, S.J. O'Brien, D.G. Blair, and G.F. Vande Woude. 1986. Mechanism of met oncogene activation. *Cell* 45:895-904.

37. Park, M., M. Dean, K. Kaul, M.J. Braun, M.A. Gonda, and G. Vande Woude. 1987. Sequence of MET protooncogene cDNA has features characteristic of the tyrosine kinase family of growth-factor receptors. *Proc.Natl.Acad.Sci.USA* 84:6379-6383.

38. Qiao, H., R. Saulnier, A. Patrzykat, N. Rahimi, L. Raptis, J.P. Rossiter, E. Tremblay, and B. Elliott. 2000. Cooperative effective of hepatocyte growth factor and fibronectin in anchorage-independent survival of mammary carcinoma cells: requirement for phosphatidylinositol 3-kinase activity. *Cell Growth Differ.* 11:123-133.

39. Rahimi, N., S. Etchells, and B. Elliott. 1996. Hepatocyte growth factor (HGF) is a copper-binding protein: a facile probe for purification of HGF by immobilized Cu(II)- affinity chromatography. *Protein Expression & Purification* 7:329-333.

40. Rahimi, N., W. Hung, R. Saulnier, E. Tremblay, and B. Elliott. 1998. c-Src kinase activity is

required for hepatocyte growth factor-induced motility and anchorage-independent growth of mammary carcinoma cells. *J.Biol.Chem.* 273:33714-33721.

41. Rahimi, N., E. Tremblay, L. McAdam, M. Park, R. Schwall, and B. Elliott. 1996. Identification of a hepatocyte growth factor autocrine loop in a murine mammary carcinoma. *Cell Growth Differ.* 7:263-270.

42. Rubin, J.S., A.M. Chan, D.P. Bottaro, W.H. Burgess, W.G. Taylor, A.C. Cech, D.W. Hirschfield, J. Wong, T. Miki, P.W. Finch, and et al. 1991. A broad-spectrum human lung fibroblast-derived mitogen is a variant of hepatocyte growth factor. *Proc.Natl.Acad.Sci.USA* 88:415-419.

43. Schaefer, L.K., S. Wang, and T.S. Schaefer. c-Src activates the DNA binding and transcriptional activity of Stat3 molecules: serine 727 is not required for transcriptional activation under certain circumstances. *Biochemical & Biophysical Research Communications.1999.Dec.20.* 266:481-487.

44. Schmidt, C., F. Bladt, S. Goedecke, V. Brinkmann, W. Zschiesche, M. Sharpe, E. Gherardi, and C. Birchmeier. 1995. Scatter factor/hepatocyte growth factor is essential for liver development. *Nature* 373:699-702.

45. Seki, T., M. Hagiya, M. Shimonishi, T. Nakamura, and S. Shimizu. 1991. Organization of the human hepatocyte growth factor-encoding gene. *Gene* 102:213-219.

46. Siefried, J. M., Weissfeld, L. A., Singh-Kaw, P., Weyant, R. J., Testa, J. R., and Landreneau, R. J. Association of immunoreactive hepatocyte growth factor with poor survival in resectable non-small cell lung cancer. Cancer Research 57, 433-439. 1997.

47. Tuck, A.B., M. Park, E.E. Sterns, A. Boag, and B.E. Elliott. 1996. Coexpression of hepatocyte growth factor and receptor (Met) in human breast carcinoma. *Am.J.Path.* 148:225-232.

48. Turkson, J., T. Bowman, R. Garcia, E. Caldenhoven, R.P. De Groot, and R. Jove. 1998. Stat3 activation by Src induces specific gene regulation and is required for cell transformation. *Mol.Cell.Biol.* 18:2545-2552.

49. Vlahos, C.J., W.F. Matter, K.Y. Hui, and R.F. Brown. 1994. A specific inhibitor of phosphatidylinositol 3-kinase, 2-(4- morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002). *J.Biol.Chem.* 269:5241-5248.

50. Webster, M.A., R.D. Cardiff, and W.J. Muller. 1995. Induction of mammary epithelial hyperplasias and mammary tumors in transgenic mice expressing a murine mammary tumor virus/activated c-src fusion gene. *Proc.Natl.Acad.Sci.USA* 92:7849-7853.

51. Weidner, K.M., N. Arakaki, G. Hartmann, J. Vandekerckhove, S. Weingart, H. Rieder, C. Fonatsch, H. Tsubouchi, T. Hishida, Y. Daikuhara, and et al. 1991. Evidence for the identity of human scatter factor and human hepatocyte growth factor. *Proc.Natl.Acad.Sci.USA* 88:7001-7005.

Figure 1 - See Figure 1 for legend

Figure 2. Treatment with the c-Src family kinase inhibitor PP2 decreases HGF mRNA level. Prestarved SP1 cells were incubated with the Src family kinase inhibitor PP2 at the concentrations indicated. After 24 hours, cells were lysed and total RNA was extracted with TriZol reagent (Life Technology) according to manufacturer's instructions. cDNA was synthesized from 1 μg of total RNA using AMV reverse transcriptase. The amount of HGF mRNA in each sample was quantitated using RT-PCR with HGF-specific primers and primers for GUS B (25 cycles of 94°C, 1 min, 55°C, 1 min, 72°C, 1 min). The amount of HGF mRNA was normalized to GUS B mRNA, and the level of HGF mRNA expression in each group was expressed as a percentage of that in untreated (control) cells. Values represent the mean of two experiments +/- range.

Figure 3. c-Src kinase activity modulates HGF mRNA and protein levels in SP1 cells.
Panel A: SP1 cells transfected with dominant negative Src (SRC-RF) or activated Src (SRC-Y527F) or empty vector (control) were prestarved overnight. PP2 (40 μM) was added to one plate of SP1 cells and incubated for an additional 24 h. A nonmalignant breast epithelial cell line HC11 was used as a negative control. Total RNA was isolated, and the amount of HGF mRNA in each sample was quantitated using RT-PCR and normalized to GUS B mRNA as described in Figure 1. The level of HGF mRNA expression in each group was expressed as a percentage of that in untreated (control) cells. Values represent the mean of two experiments +/- range.

Panel B: Serum-free conditioned media were collected for 24 h from HC11 cells, PP2-treated SP1 cells, and SP1 cells transfected as in Panel A. HGF protein from the conditioned media was purified using copper (II) affinity chromatography (71). The fraction containing HGF protein was concentrated in Microcon concentrators and subjected to denaturing SDS-PAGE. Recombinant HGF (100 ng) was included in one lane as a control. After electrophoresis, the proteins were transferred onto nitrocellulose and the blot was probed with anti-HGF antibody. Immunoreactive bands were revealed using ECL.

Figure 4. *HGF* Transcription is regulated by c-Src kinase in Cos-1 fibroblasts and SP1 carcinoma cells.
SP1 (Panel A), and Cos-1 (Panel B) cells were co-transfected with the *HGF*-luciferase reporter (2.7 HGF-luc) and activated c-Src (SRC-Y527F), dominant negative c-Src (SRC-RF) or an empty vector (control). A β-galactosidase expression plasmid was co-transfected in each group for normalization to account for differences in transfection efficiency. After 48 hours, cells were lysed and the lysate was used for luciferase assays. Luciferase activity was measured as light emission and detected by luminometer. The amount of luciferase activity in each sample was normalized to transfection efficiency as determined by β-galactosidase activity as an internal control. Luciferase activity of each sample was determined and expressed as fold-induction of that in control cells transfected with empty vector. Values represent the mean +/- SD of triplicate samples. The experiments were done three times with similar results.

Figure 5. c-Src kinase responsiveness of *HGF* transcription requires the -254 to -70 bp region of the *HGF* promoter.

Panel A: SP1 cells were co-transfected with the *HGF*-luciferase reporter (2.7 HGF-luc), reporter constructs containing various deletions of the HGF promoter (1.2, 0.8, 0.5, 0.3, 0.1 and 0.5Δ), or constructs containing internal deletion of regions between -254 and -70 (Δ1), -1231 and -755 (Δ2), or both (ΔΔ), and activated c-Src (SRC-Y527F), dominant negative c-Src (SRC-RF) or an empty expression vector (control). Luciferase activity was determined and expressed as a percentage of control cells as described in Figure 4. Values represent mean +/- SD of triplicate samples. The experiments were done three times using two different preparations of plasmid DNA with similar results.
Panel B: Schematic representation of the wildtype *HGF* reporter construct and the corresponding internal deletion mutants used in Panel A. Each acronym refers to the full length (2.7 kb) or truncated promoter sequence upstream of the transcriptional start site (indicated by arrow).

Figure 6 Proposed Organization of the *HGF* Promoter

The HGF promoter sequence was scanned for known consensus binding sequence for transcription factors. A number of binding sites were identified and are shown schematically here. Chicken ovalbumin upstream promoter-transcription factor (COUP-TF), estrogen receptor (ER), Sp1 and CAATT/enchancer binding protein (C/EBP) have been shown to affect HGF expression in various cell lines. Consensus sites for ETS, AP-1, TGF-β inhibitory element binding protein (TIE BP), IL-6 responsive element binding protein (IL-6 RE BP), Lef-1 and Stat1, 3, 5 are found but their roles have not been confirmed. Two regions of the HGF promoter that are responsive to c-Src kinase activity are highlighted.

Figure 7. Stat3 induces *HGF* transcription in co-operation with activated c-Src.
SP1 carcinoma cells (Panel A) and HC11 mammary epithelial cells (Panel B) were co-transfected with the 2.7 HGF-luc reporter and activated c-Src (SRC-Y527F) or an empty vector (control), in combination with varying amounts of Stat3. Luciferase activity was determined and expressed as a percentage of that in control cells as described in see Figure 4. Values represent the mean +/- SD of triplicate samples. The experiments were done twice with similar results.

Figure 8. Stat3 synergizes with activated c-Src to induce *HGF* transcription.
SP1 cells were co-transfected with the 2.7 HGF-luciferase reporter, or an internal deletion mutant (Δ1 HGF-luc), and a combination of activated Src (SRC-Y527F) and Stat3 as indicated in the figure. Transfections and luciferase assays were performed as described in Figure 1B. Values represent the mean +/- SD of triplicate samples. The experiments were done four times with similar results.

Figure 9. c-Src kinase activity regulates phosphorylation of tyrosine residue 705 of Stat3
SP1 cells transfected with SRC-RF or SRC Y527F, or untransfected SP1 cells, were lysed. Equal amounts of proteins from each cell lysate were subjected to denaturing SDS-PAGE. The proteins were then transferred onto nitrocellulose and the blot was probed with antibody specific for phosphotyrosine 705 of Stat3 (Panel A). The blot was subsequently reprobed with anti-Stat3 (pan) antibody (Panel B).

Figure 10. c-Src kinase activity regulates binding of Stat3 to the Src-responsive region of the *HGF* promoter.
Nuclear extracts were prepared from SP1 cells transfected with SRC-RF, or SRC-Y527F, or untransfected cells. Equal amounts of each nuclear extract were used in binding studies with radiolabelled probes containing either the -110 (Panel A) or the -149 region (Panel B) of the *HGF* promoter. Ten-fold molar excess of an unlabelled probe containing the -110, -149 or a non-specific sequence (NS), respectively, was included in the binding reaction where indicated. The gel was fixed, dried, and analyzed using a Storm PhosphorImager as described in Materials and Methods. The arrow indicates the position of the protein-DNA complex.

Figure 11. Stat3 forms part of the DNA-protein complex at both the -110 and -149 consensus sites. Nuclear extracts were prepared from SP1 cells. For supershift assays, nuclear extracts were incubated with anti-Stat3, Stat5A or Stat5B antibody on ice for 30 minutes prior to EMSA analysis. After incubation with labelled -110 (Panel A), or -149 (Panel B), probes, the reaction was subjected to non-denaturing PAGE. The asterisk indicates the position of supershift band.

Figure 12. Inhibition of Ras family members causes a reduction in HGF mRNA level.
SP1 cells were plated at 80% confluence and prestarved overnight. Cells were then treated with farnesyl transferase inhibitor B956 (10 μM). At indicated time point, cells were lysed with TriZol reagent and total RNA was extracted. cDNA was synthesized and RT-PCR reactions were performed as described in Figure 2. The amount of HGF mRNA expression compared to control was plotted and is shown.

Figure 13. Activated Ras induces HGF transcription through PI 3-kinase dependent pathway.
SP1 cells were co-transfected with the *HGF*-luciferase reporter (2.7 HGF-luc), and expression plasmid for various Ras mutants, namely the activated Ras (Ras V12), inactive Ras (Ras V12 A38), activator of the Raf/ERK pathway (Ras V12 E38) and activator of the PI 3-kinase pathway (Ras V12 C40) or an empty expression vector (control). Luciferase activity was determined and expressed as a percentage of control cells as described in Figure 4. Values represent mean +/- SD of triplicate samples.

Figure 14. Inhibition of PI 3-kinase reduces *HGF* transcription.
SP1 cells were transfected with a reporter plasmid containing the 2.7 kb fragment of the HGF promoter driving expression of the luciferase gene (2.7 HGF-luc). A β-galactosidase expression plasmid was co-transfected in each group for normalization to account for differences in transfection efficiency. After 24 h of incubation, LY294002 was added at the concentrations indicated, and the cells were incubated for an additional 24 h, lysed and assayed for luciferase activity as in Figure 4. Luciferase activity of each sample was expressed as percentage of control cells transfected with empty vector. Values represent the mean +/- SD of triplicate samples. The experiment was done twice with similar results.

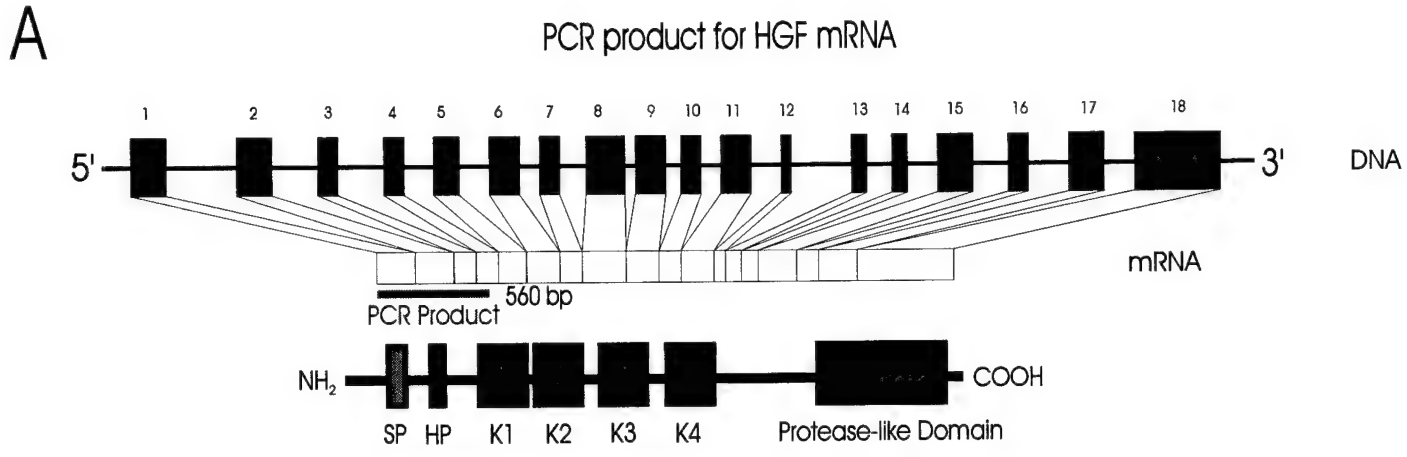


Figure 1:

Panels A Design of PCR primers for HGF: Primers were designed to overlap more than one exon and to crossreact between mouse and human. HGF primers: 5' (sense) TGT CGC CAT CCC CTATGC AG (corresp. to bases 69-88 of hHGF); 3' (antisense) TCA ACT TCT GAA CAC TGA GG. (corresp. to bases 610-629 of hHGF).

Panel B Detection by RT-PCR of HGF mRNA in mouse breast carcinoma cell line, SP1: cDNA was prepared from 1 μg of total RNA, and subjected to RT-PCR of 25 cycles of: 1 min at 95°C (denaturing), 1 min at 55°C (annealing), and 1 min at 72°C (elongation). Lane 1: DNA molecular size markers; lane 2, 206 bp marker; lane 3, PCR product of HGF.

Figure 2
Effect of c-Src kinase inhibitor PP2 on HGF mRNA levels in SP1 cells

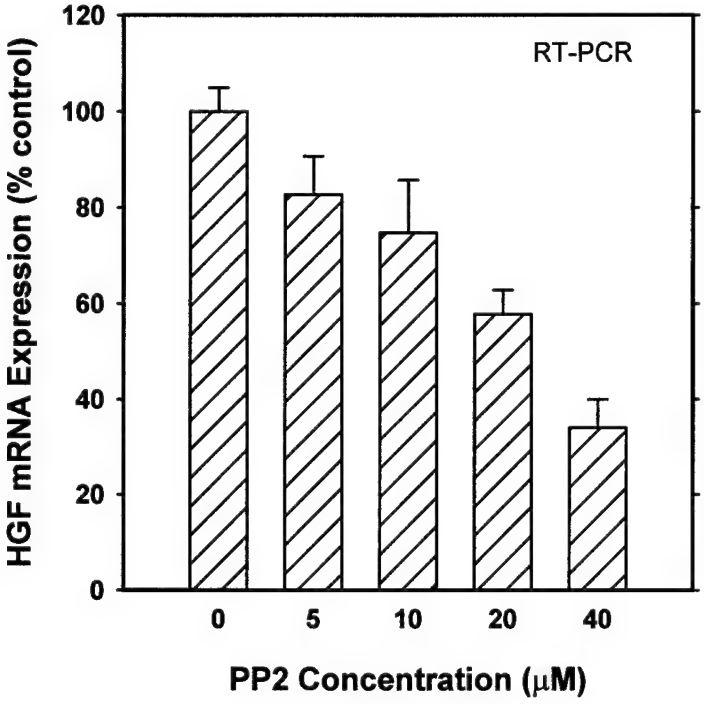


Figure 3

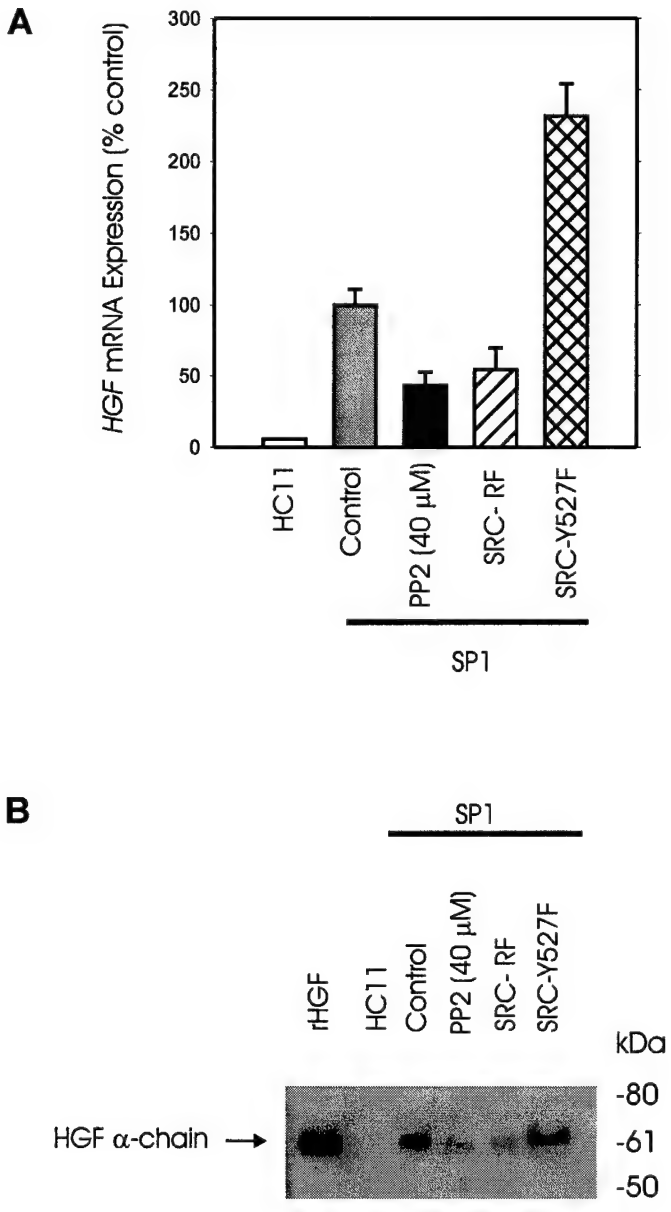
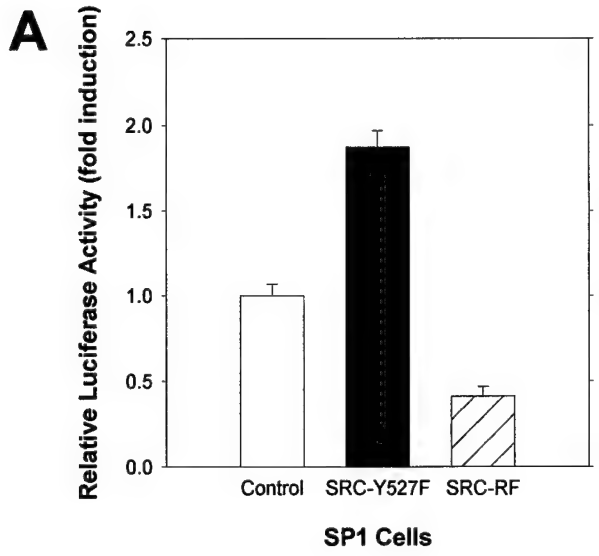

A
HGF mRNA Expression (% control)
300
250
200
150
100
50
0
HC11
Control
PP2 (40 μM)
SRC- RF
SRC-Y527F
SP1
B
SP1
rHGF
HC11
Control
PP2 (40 μM)
SRC- RF
SRC-Y527F
kDa
HGF α-chain
-80
-61
-50

Figure 4


A
Relative Luciferase Activity (fold induction)
2.5
2.0
1.5
1.0
0.5
0.0
Control
SRC-Y527F
SRC-RF
SP1 Cells

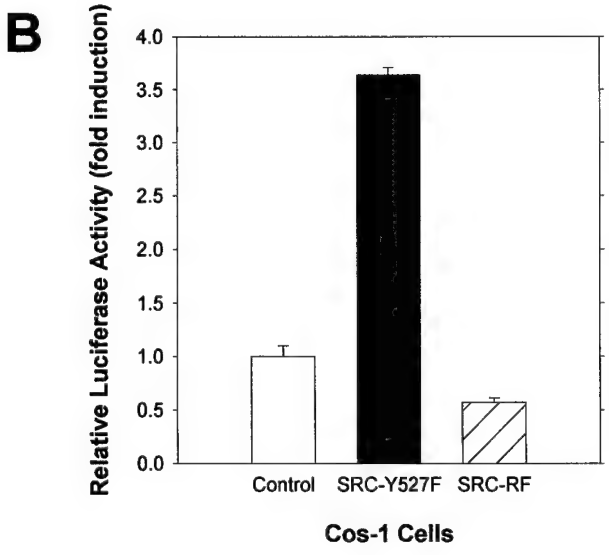
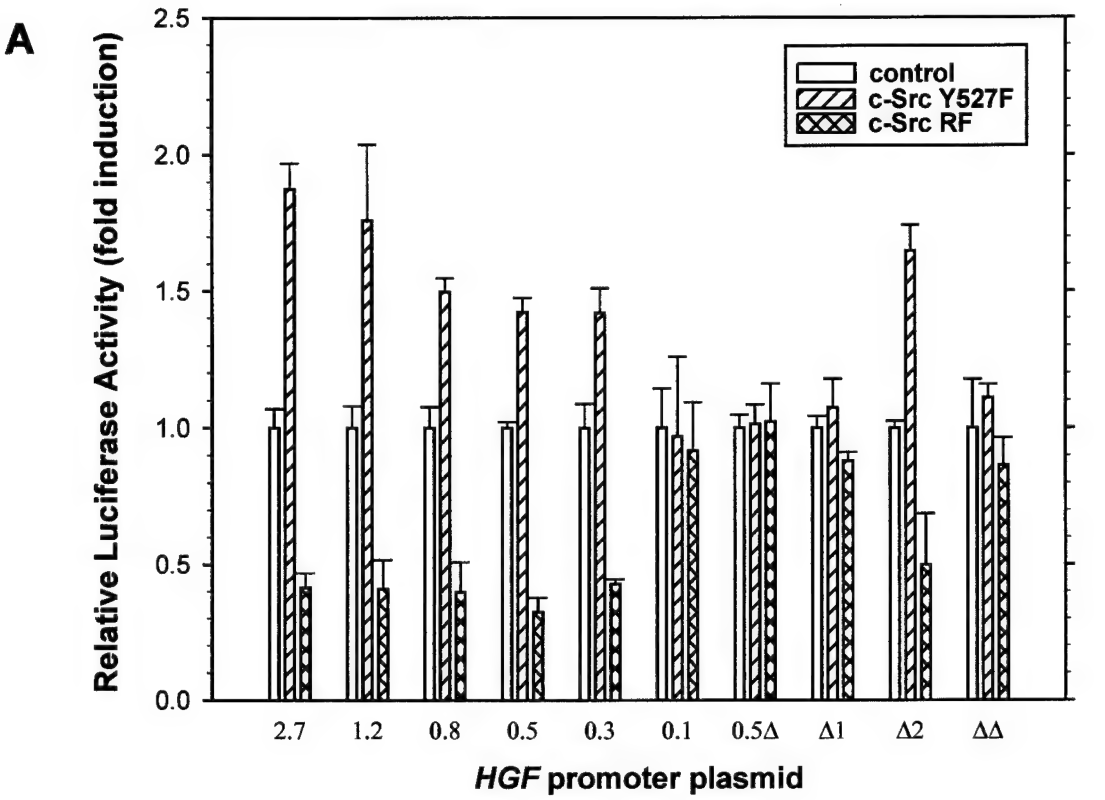

B
Relative Luciferase Activity (fold induction)
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Control
SRC-Y527F
SRC-RF
Cos-1 Cells

Figure 5


A
Relative Luciferase Activity (fold induction)
2.5
2.0
1.5
1.0
0.5
0.0
control
c-Src Y527F
c-Src RF
2.7
1.2
0.8
0.5
0.3
0.1
0.5Δ
Δ1
Δ2
ΔΔ
HGF promoter plasmid

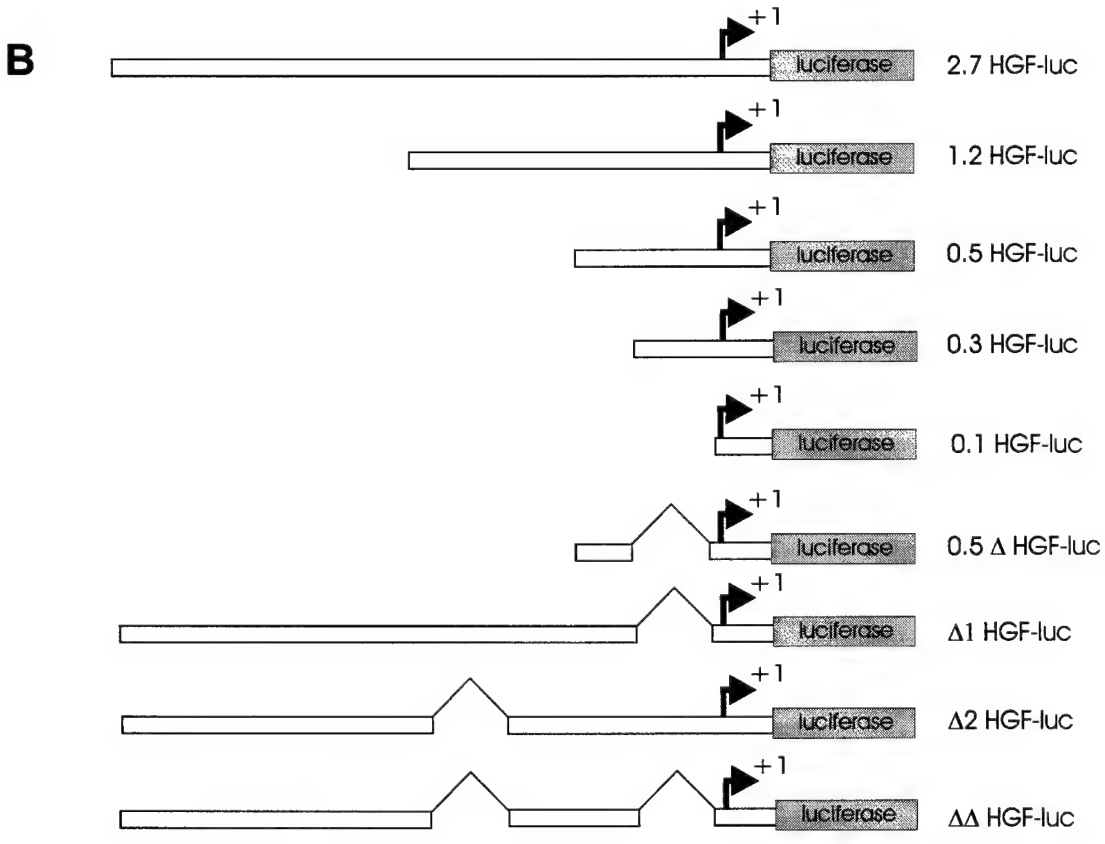

B
+1
luciferase
2.7 HGF-luc
1.2 HGF-luc
0.5 HGF-luc
0.3 HGF-luc
0.1 HGF-luc
0.5 Δ HGF-luc
Δ1 HGF-luc
Δ2 HGF-luc
ΔΔ HGF-luc

Figure 6
Proposed Organization of *HGF* Promoter Region

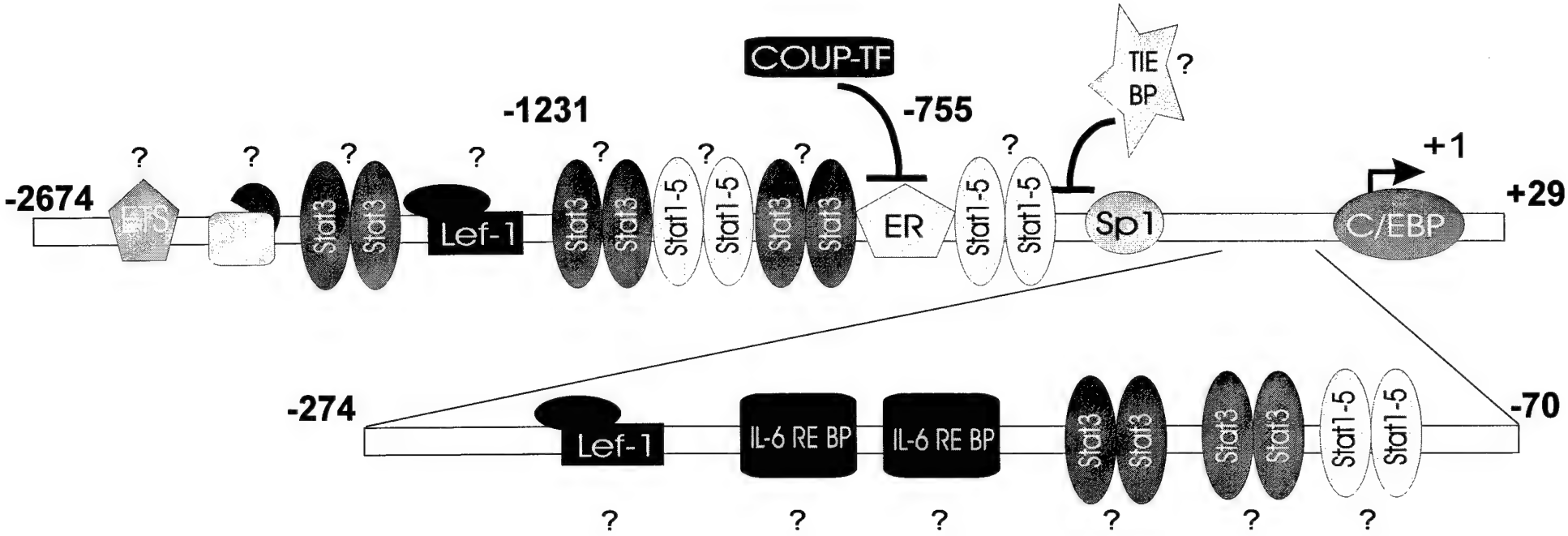
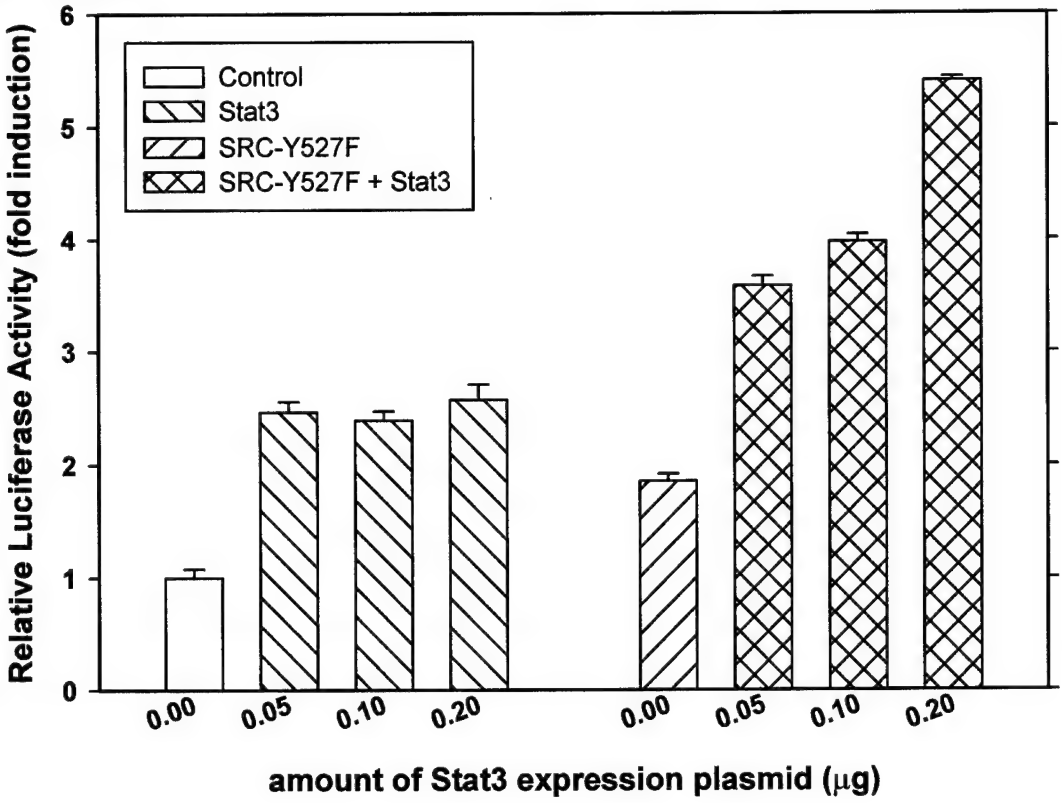


Figure 7

A. SP1 cells



B. HC11 cells

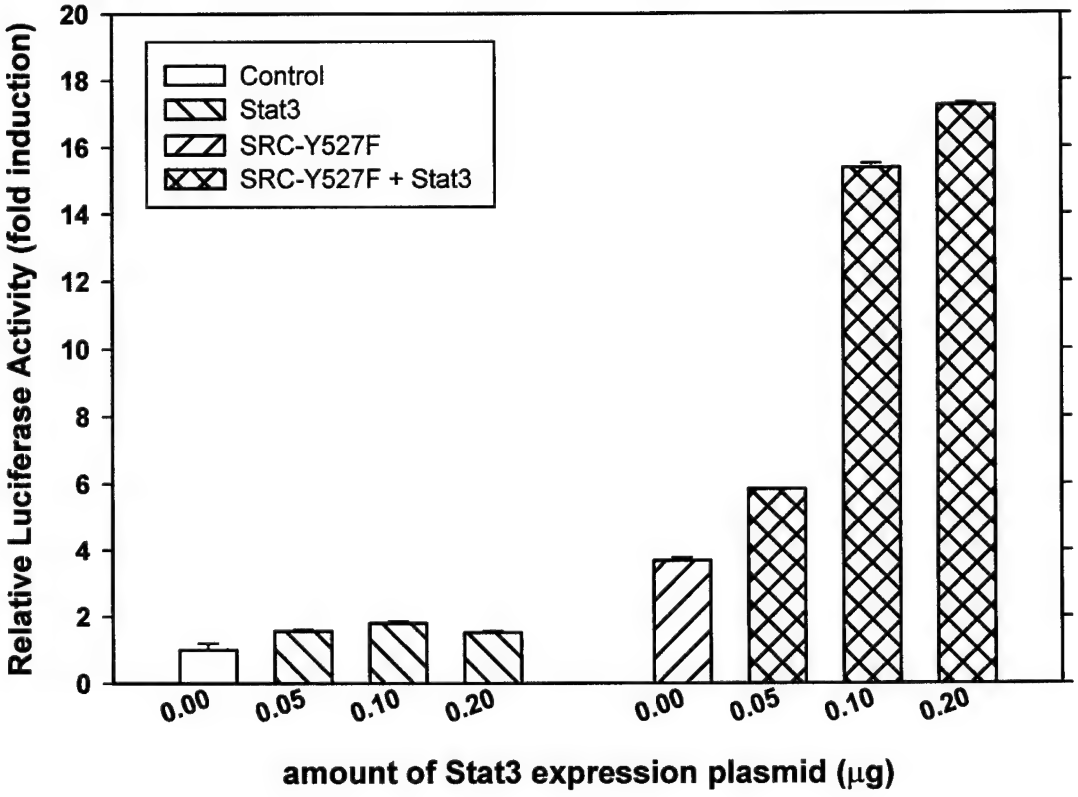


Figure 8

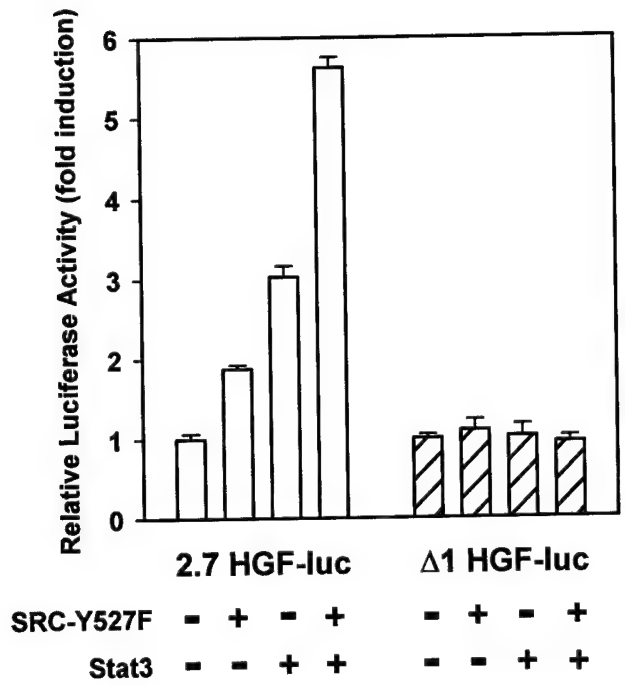

Relative Luciferase Activity (fold induction)
0
1
2
3
4
5
6
2.7 HGF-luc
Δ1 HGF-luc
SRC-Y527F - + - + - + - +
Stat3 - - + + - - + +

Figure 9

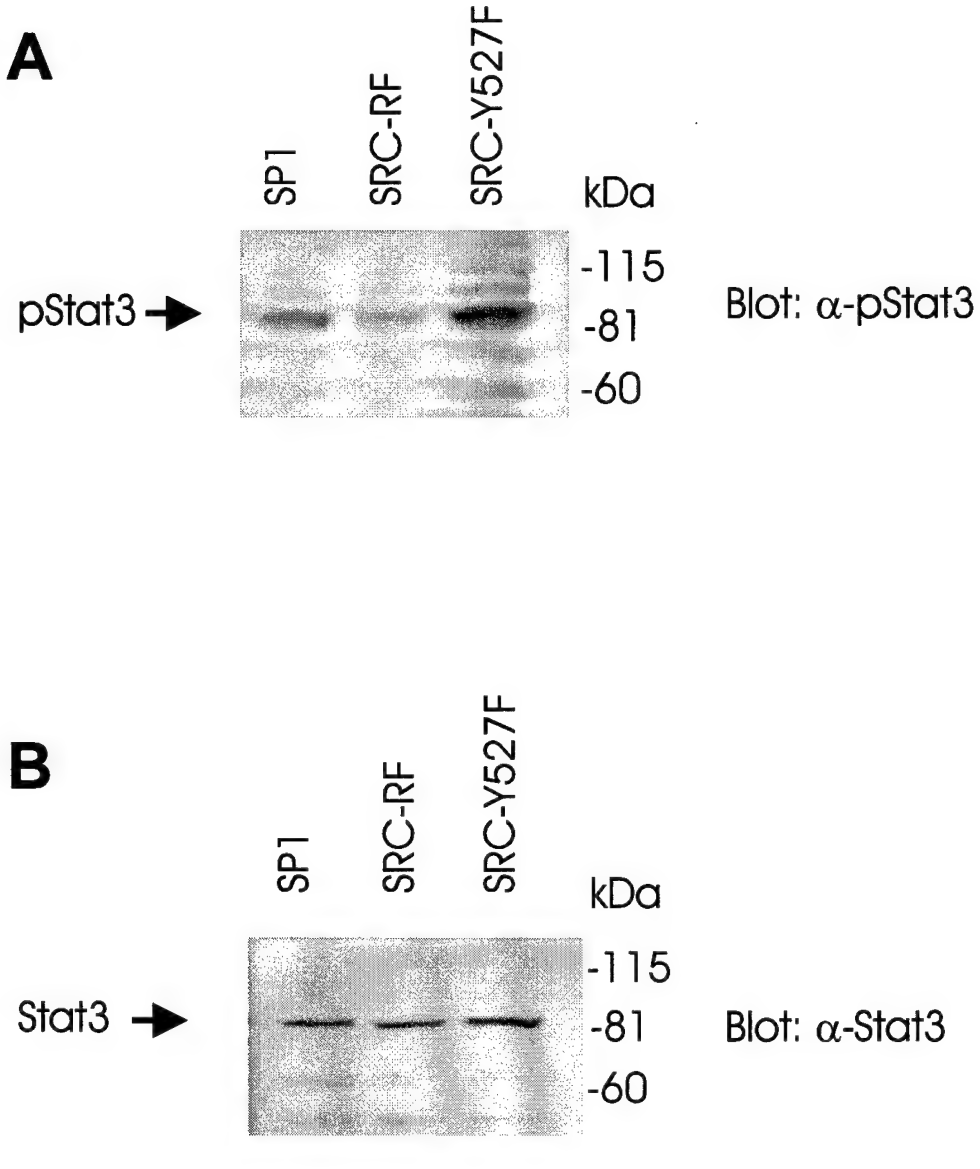
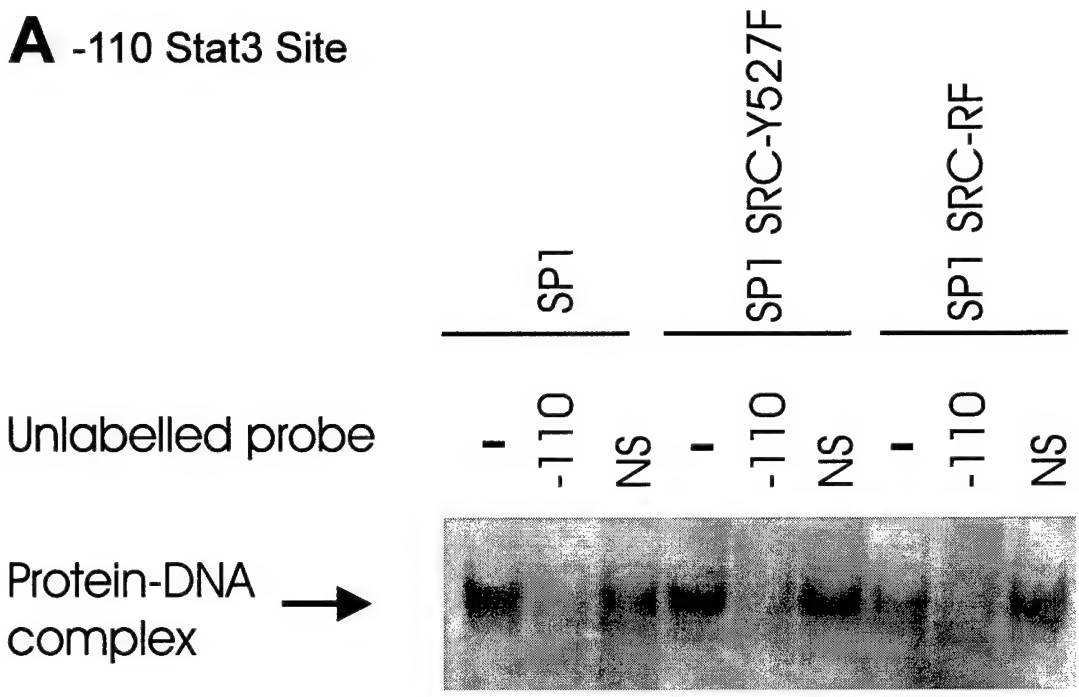

A
SP1
SRC-RF
SRC-Y527F
kDa
-115
-81
-60
pStat3
Blot: α-pStat3
B
SP1
SRC-RF
SRC-Y527F
kDa
-115
-81
-60
Stat3
Blot: α-Stat3

Figure 10

A -110 Stat3 Site



B -149 Stat3 Site

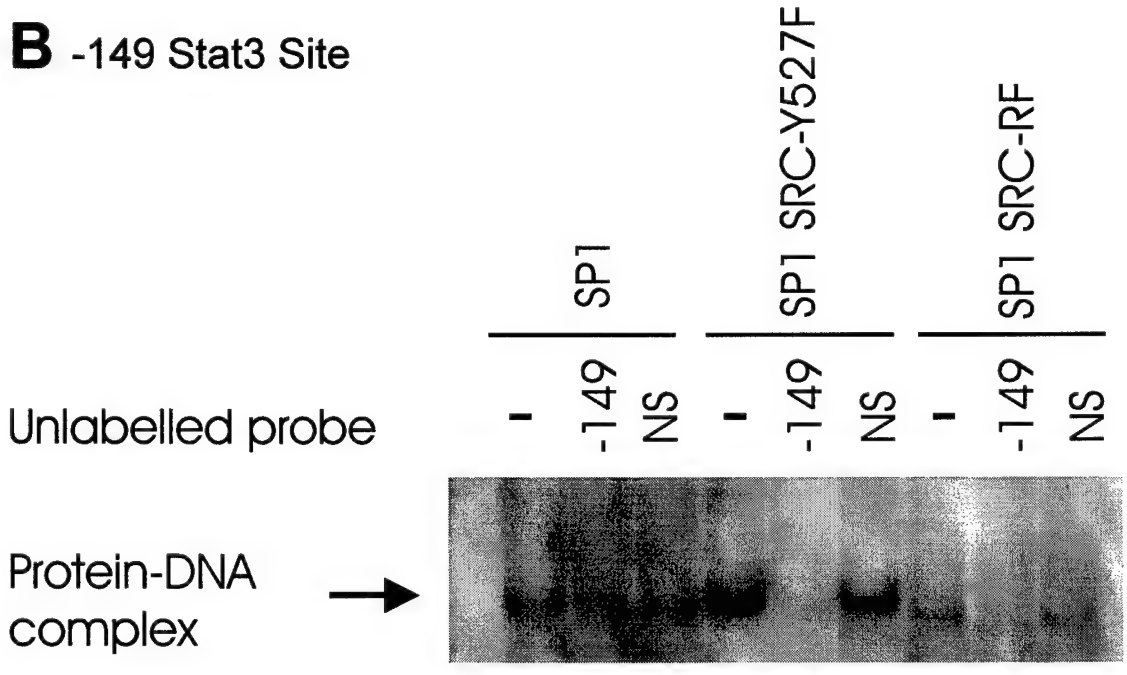
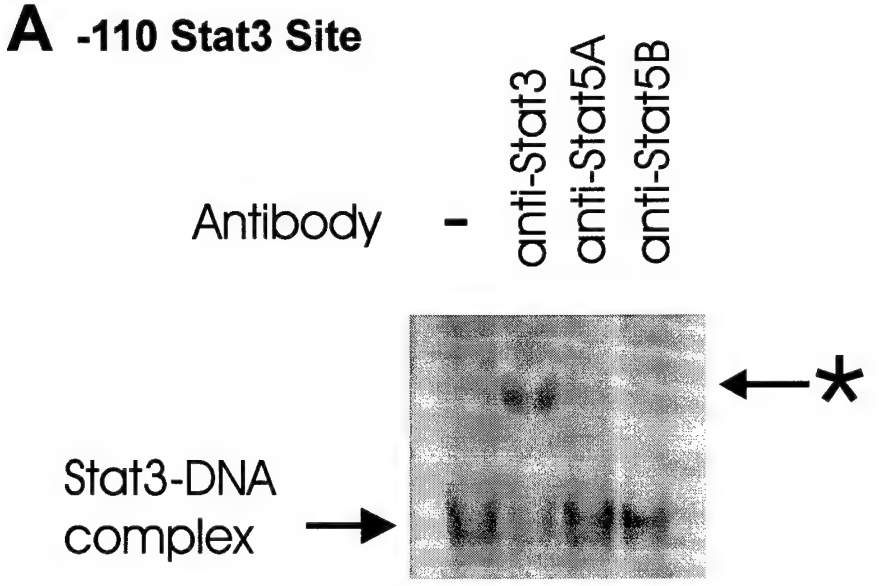


Figure 11


A -110 Stat3 Site
Antibody
-
anti-Stat3
anti-Stat5A
anti-Stat5B
Stat3-DNA complex
*

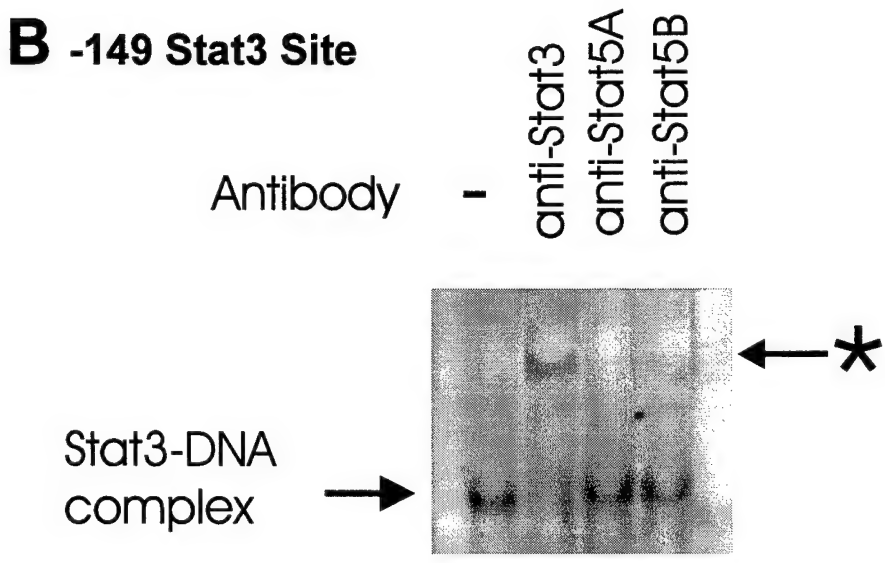

B -149 Stat3 Site
Antibody
-
anti-Stat3
anti-Stat5A
anti-Stat5B
Stat3-DNA complex
*

Figure 12
Effect of Ras inhibitor B956 on HGF mRNA levels in SP1 cells

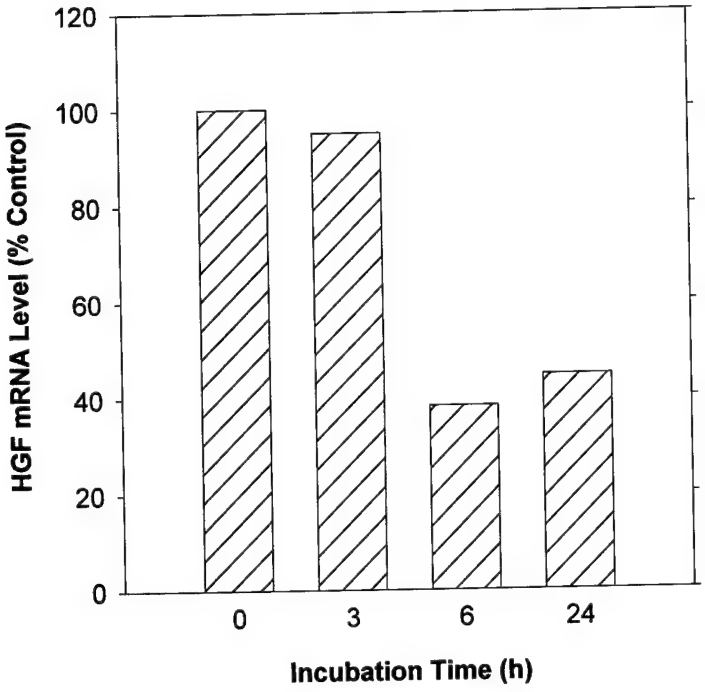


Figure 13
Effect of Ras Mutants on *HGF* Promoter Activity

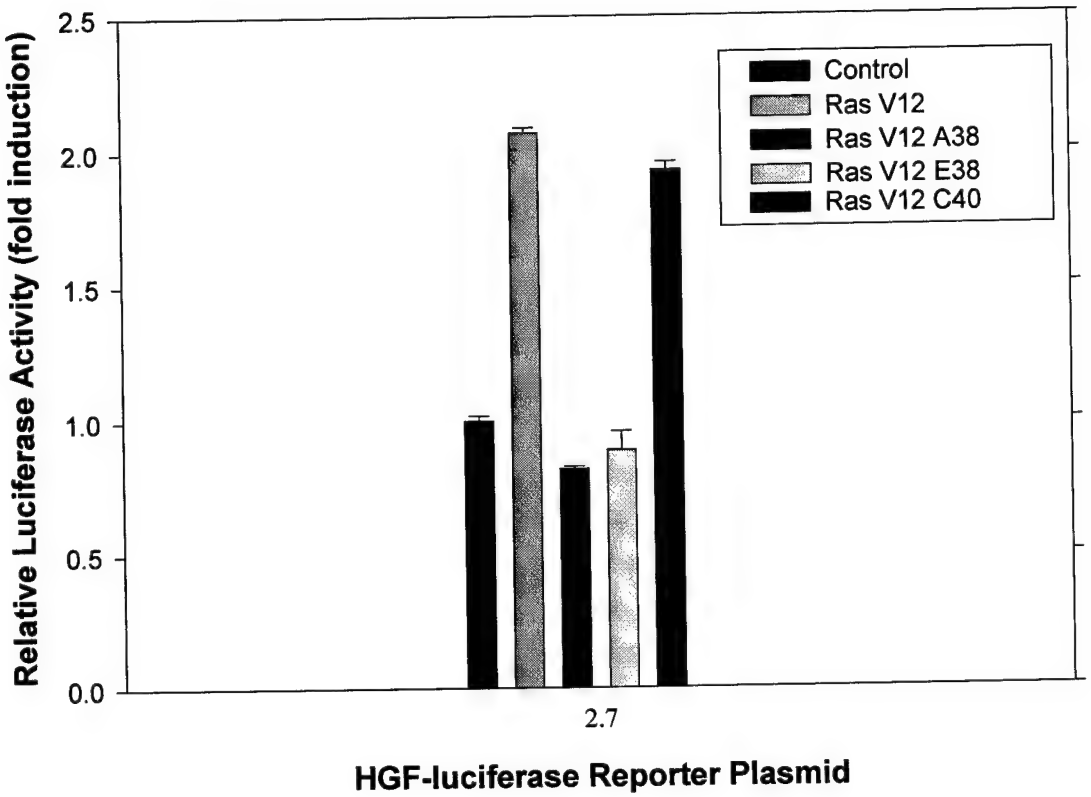
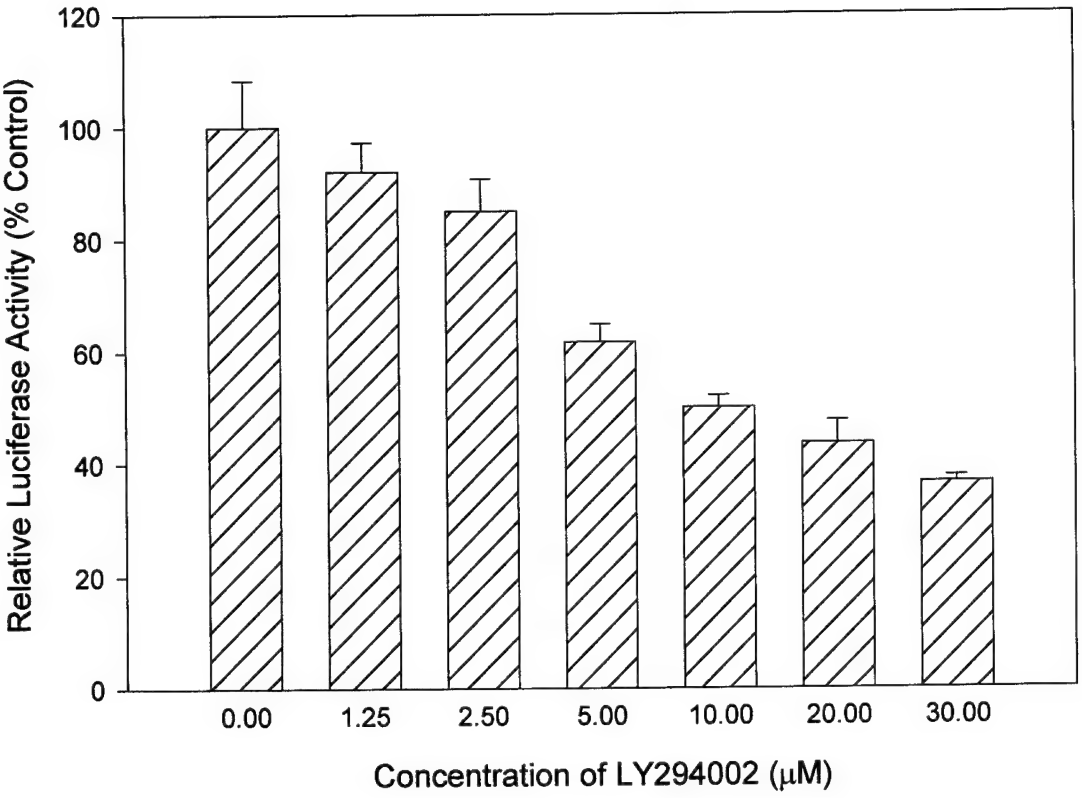


Figure 14
Effect of PI 3-KKinase Inhibitor LY294002 on *HGF* Promoter Activity in SP1 cells



Summary of key research accomplishment

- Identification of c-Src responsive region in HGF promoter
- Identification of Stat3 as downstream target of c-Src
- Demonstration of c-Src and Stat3 synergistic regulation of HGF transcription
- Identification of Stat3 as part of DNA-protein complex binding to the HGF promoter
- Determination of the mechanism by which c-Src regulates HGF expression through Stat3 tyrosine phosphorylation and DNA binding affinity
- Identification of Ras as a signalling molecule regulating HGF expression and possible involvement of PI 3-kinase as downstream target of Ras
- Establishment of stable SP1 cell lines expressing c-Src, activated c-Src, dominant negative c-Src
- Establishment of stable HC11 cell lines expressing c-Src, activated c-Src, dominant negative c-Src and FLAG-tagged Stat3

List of Abstracts Presented

Hung, W., and B. Elliott. 2000. A novel c-Src kinase/Stat3 pathway regulates hepatocyte growth factor expression in breast carcinoma cells. Signal Transduction Pathways and Regulation of Gene Expression as Therapeutic Targets Conference, Luxembourg city, Luxembourg.

Hung, W. 2000. A c-Src kinase/Stat3 pathway regulates hepatocyte growth factor expression in breast carcinoma cells. Invited Oral Presentation at the Institut Curie, Paris, France.

Hung, W., and B. Elliott. 2000. A novel c-Src kinase/Stat3 pathway regulates hepatocyte growth factor expression in breast carcinoma cells. Era of Hope: Breast Cancer Research Conference, Atlanta, Georgia

Abstract

A NOVEL c-SRC TYROSINE KINASE/STAT3 PATHWAY REGULATES HEPATOCYTE GROWTH FACTOR EXPRESSION IN MAMMARY CARCINOMA CELLS

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Aberrant expression of hepatocyte growth factor (HGF) and its receptor Met occurs in many carcinoma cell types. However, in nonmalignant tissues, expression of HGF is tightly regulated and is expressed primarily in the stromal cells, while epithelial cells express Met but not HGF. We have previously identified co-expression of Met and HGF in the invasive tumor front of human breast carcinomas, and secretion of HGF and constitutive activation of Met and downstream molecules in a murine breast carcinoma cell line SP1 (Cell Growth Diff. 7:263-270, 1996). These observations suggest the presence of an HGF autocrine loop in breast carcinoma cells, which may confer survival and growth advantage during tumor progression and metastasis. We previously showed that c-Src tyrosine kinase is activated in SP1 carcinoma cells, and its activity is required for HGF-induced motility and anchorage-independent growth (JBC 273: 33714-21, 1998). It has been shown that c-Src kinase activity is critical in regulating the expression of some growth factors. Therefore, in this study, we examined the role of c-Src in HGF expression in establishment of an HGF autocrine loop in breast carcinoma cells. We found that HGF mRNA and protein levels were reduced when SP1 cells expressed a dominant negative form of c-Src (K295R, Y527F). Expression of an activated form of c-Src (Src Y527F) increased transcription from the *HGF* promoter, while expression of the dominant negative c-Src had the opposite effect. Using a series of reporter constructs containing deletions of the *HGF* promoter linked to the luciferase gene, we determined the location of sites that may be important for c-Src responsiveness of HGF expression. We found that in Cos-1 cells two regions of the *HGF* promoter were responsive to c-Src kinase activity. The first region, between -1231 and -755, accounted for one third of the c-Src responsiveness of the *HGF* promoter. The second region, between -254 and -70, provided the remaining amount of c-Src responsiveness. Interestingly, in SP1 breast carcinoma cells, only the latter site (-254 to -70) has a significant effect on c-Src induced HGF expression. Both regions showed a consensus sequence for Stat3 binding. Deletion of these Stat3 binding sites eliminated c-Src responsiveness of the *HGF* promoter. Furthermore, co-expression of c-Src Y527F and Stat3 synergistically increased *HGF* promoter activity. Expression of activated c-Src also induced tyrosine phosphorylation and DNA binding of Stat3, whereas expression of the dominant negative c-Src mutant had the reverse effect. Together our data indicate that c-Src kinase regulates HGF expression in mammary carcinoma cells through the activation of Stat3 protein. This observation suggests that activation of the c-Src tyrosine kinase/Stat3 pathway promotes the establishment of an HGF autocrine loop in breast cancer.
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A c-Src kinase/Stat3 pathway regulates hepatocyte growth factor expression in breast carcinoma cells.

Over-expression of HGF and Met occurs in many types of cancer including breast. We have previously shown co-expression of Met and HGF in the invasive tumor front of breast carcinoma, and secretion of HGF and constitutive activation of Met and downstream molecules in the murine breast carcinoma cell line, SP1. These observations suggest the presence of an HGF autocrine loop in breast carcinoma cells, which confers survival and growth advantage to carcinoma cells during tumor progression and metastasis. The c-Src non-receptor tyrosine kinase is activated in SP1 carcinoma cells and is critical in regulating the expression of many genes. In this study we examined the role of c-Src in HGF expression and establishment of an HGF autocrine loop in breast carcinoma cells. We found that HGF mRNA and protein levels were reduced when SP1 cells expressed dominant negative c-Src. Expression of activated c-Src increased transcription from the *HGF* promoter while dominant negative c-Src had the opposite effect. We found that in SP1 cells the region between -254 and -70 was required for the c-Src responsiveness of the *HGF* promoter. This region contains two putative consensus sequences for Stat3 and is required for c-Src responsiveness of the *HGF* promoter. Co-expression of activated c-Src and Stat3 synergistically induced *HGF* promoter activity. Cells expressing activated c-Src showed increased tyorsine phosphorylation and DNA binding affinity of Stat3 (but not Stat1, -5A or -5B), whereas the dominant negative c-Src mutant had the opposite effect. Together our data indicate that c-Src kinase regulates *HGF* transcription and protein expression in mammary carcinoma cells through activation of the Stat3 transcription factor, and thereby promotes a tumorigenic phenotype.